J. Clin. Chem. Clin. Biochem.
Vol. 23, 1985, pp. 821–828

Isolation and Characterization of Porcine Leukocyte Elastase

Leukocyte Elastase-Inhibitor Complexes in Porcine Blood, II[1])

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(Received July 1/October 14, 1985)

Summary: Porcine leukocyte elastase[1]) was purified from granulocytes by chelating chromatography on copper chelate Sepharose and by ion exchange chromatography on CM-Sepharose. Thus an enzyme preparation with a specific activity (substrate: MeOSuc(Ala)$_2$ProValNan)[2]) of 89.3 U/mg protein was obtained.

Dodecyl sulphate gel electrophoresis revealed one protein band corresponding to a molecular mass of 27 kDa. The amino acid composition was determined and isoleucine was identified as the only N-terminal amino acid residue.

The bimolecular velocity constant for the inhibition by diisopropyl fluorophosphate was determined as 2000 $l \times mol^{-1} \times min^{-1}$. The dissociation constants, K_i, of the complexes of porcine leukocyte elastase with various inhibitors were calculated. The kinetic constants for the elastase-catalysed hydrolysis of MeOSuc-(Ala)$_2$ProValNan, Suc(Ala)$_2$ValNan and Suc(Ala)$_3$Nan were determined, as well as the kinetic constants of the inactivation of leukocyte elastase by active site mapping reagents. Detergents such as Triton X-100, Tween 20 and Brij 35, as well as porcine serum albumin, activated the procine leukocyte elastase preparation.

Isolierung und Charakterisierung der Elastase aus Leukocyten des Schweins
Leukocyten-α_1-Proteinaseinhibitor-Komplexe in Schweineblut, II. Mitteilung

Zusammenfassung: Elastase[2]) aus Schweineleukocyten wurde mit Hilfe der Chelatchromatographie an Kupferchelat-Sepharose und Ionenaustauschchromatographie an CM-Sepharose aus Schweine-Granulocyten isoliert. Die erhaltene Enzympräparation hat eine spezifische Aktivität (Substrat: MeOSuc(Ala)$_2$ProValNan)[3]) von 89,3 U/mg Protein. In der Dodecylsulfat-Gelelektrophorese wurde eine Proteinbande erhalten, die einer Molekülmasse von 27 kDa entspricht. Die Aminosäurezusammensetzung wurde bestimmt; Isoleucin wurde als einzige N-terminale Aminosäure identifiziert.

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[1]) I. Communication l. c. (15).

[2]) Enzymes: Leukocyte elastase from polymorphonuclear granulocytes (EC 3.4.21.37, formerly EC 3.4.21.11).

[3]) Abbreviations:
MeOSuc(Ala)$_2$ProValNan:
N$^{\alpha}$-methoxysuccinyl-(*L*-alanyl)$_2$-*L*-prolyl-*L*-valine-*p*-nitroanilide
Suc(Ala)$_2$ValNan:
N$^{\alpha}$-succinyl-*L*-(*L*-alanyl)$_2$-*L*-valine-*p*-nitroanilide
Suc(Ala)$_3$Nan:
N$^{\alpha}$-succinyl-(*L*-alanyl)$_2$-*L*-alanine-*p*-nitroanilide
HEPES:
N-2-hydroxyethylpiperazine-N′-2-ethane sulphonic acid
ZPheCH$_2$Br:
1-bromo-4-phenyl-3-(N-benzyloxycarbonyl)-amino-*L*-butane-2-one
Ac(Ala)$_2$ProAlaCH$_2$Cl:
N$^{\alpha}$-acetyl-*L*-alanyl-*L*-alanyl-*L*-prolyl-*L*-alanine chloromethyl ketone
Ac(Ala)$_3$AlaCH$_2$Cl:
N$^{\alpha}$-acetyl-*L*-alanyl-*L*-alanyl-*L*-alanyl-*L*-alanine chloromethyl ketone

Die Geschwindigkeitskonstante der Inhibierung des Enzyms durch Diisopropylfluorophosphat betrug 2000 $l \times mol^{-1} \times min^{-1}$. Die Dissoziationskonstante K_i der Komplexe der Elastase aus Schweineleukocyten mit verschiedenen Inhibitoren wurde bestimmt. Die kinetischen Konstanten der Hydrolyse von MeOSuc(Ala)$_2$ ProValNan, Suc(Ala)$_2$ValNan und Suc(Ala)$_3$Nan wurden gemessen, ebenso die kinetischen Konstanten der Inaktivierung der Elastase durch Reagentien, die gegen das aktive Zentrum der Elastase gerichtet sind. Detergenzien wie Triton X-100, Tween 20 und Brij 35 sowie Albumin aus Schweinserum aktivieren Elastase aus Schweineleukocyten.

Introduction

Leukocyte elastase (EC 3.4.21.37) is a serine proteinase present in lysosomal (azurophil) granules of neutrophil leukocytes (1). The enzyme digests native elastin, the elastic fibrous protein in connective tissue. There is clear evidence that leukocyte elastase may play an important role in physiological events (e. g. digestion of bacteria after phagocytosis (2)) as well as in pathophysiological processes such as degradation of kidney basement membranes in glomerulonephritis, destruction of cartilage in rheumatoid arthritis and degradation of elastin in arterial walls. Furthermore, it was recently demonstrated that elastase, liberated from leukocytes by endotoxins during sepsis, can degrade blood clotting factors (3, 4).

It is well known that α_1-proteinase inhibitor preferentially inhibits leukocyte elastase in human plasma (5). Thus, after extracellular liberation of leukocyte elastase under pathophysiological conditions this enzyme is rapidly inactivated and elastase-inhibitor complexes can be detected in the circulation. In practice, it should be possible to measure these complexes by enzyme immunoassay (4, 6). For the development of a suitable enzyme immunoassay for leukocyte elastase inhibitor complexes, we have isolated and characterized the porcine leukocyte elastase.

Materials and Methods

Chelating Sepharose and CM-Sephadex C-50 were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. MeOSuc(Ala)$_2$ProValNan, Suc(Ala)$_2$ValNan and Suc(Ala)$_3$Nan were products of Novabiochem AG, Läufelfingen, Switzerland. Servalyte 2–11, Tween 20, Brij 35, Triton X-100 and soy bean inhibitor were purchased from Serva AG, Heidelberg, FRG. Diisopropylfluoro phosphate, lima bean trypsin inhibitor, chicken egg white ovomucoid inhibitor, turkey egg white ovomucoid inhibitor, phenylmethylsulphonylfluoride and porcine serum albumin where products from Sigma chemicals, St. Louis, USA. Aprotinin was a gift of Bayer AG, Leverkusen, FRG. Human leukocyte elastase was kindly provided by *S. Neumann*, Merck, Darmstadt, FRG. Ac(Ala)$_2$ProAlaCH$_2$Cl and Ac(Ala)$_3$AlaCh$_2$Cl were gifts of *J. C. Powers*, Atlanta, USA. ZPheCH$_2$Br was kindly provided by *E. Shaw*, New York, USA.

Starting material

Fresh whole porcine blood (50 l) was used as starting material for the isolation of leukocytes. Preparation of granulocytes was performed as described by *Engelbrecht* et al. (7). The resulting leukocyte pellet was used for the purification of elastase.

Activity and inhibition measurements

To measure porcine leukocyte elastase activity the following assay system was used (wavelength: 405 nm; 25 °C; volume: 1 ml; $\varepsilon = 1020\ m^2 \times mol^{-1}$ (8): 0.98 ml 2.14 mol/l MeOSuc-(Ala)$_2$ProValNan in 0.1 mol/l HEPES buffer, pH 7.5, containing 0.5 mol/l sodium chloride were incubated at 25 °C for 5 min. Thereafter 0.02 ml elastase solution was added and the increase in absorbance per minute was read for 5 minutes.

Inhibition studies were performed according to *Starkey & Barrett* (9) with the exception that MeOSuc(Ala)$_2$ProValNan was used as substrate instead of azo-casein.

Michaelis-Menten constants were determined according to *Wilkinson* (10) after graphical inspection of the data in *Lineweaver-Burk* diagrams.

The dissociation constants, K_i, of elastase-inhibitor complexes were estimated graphically according to *Dixon* (11). Molarity of inhibitor solutions were determined by titrating trypsin or chymotrypsin solutions with inhibitor as described in l. c. (12).

Activation of porcine leukocyte elastase with detergents and porcine serum albumin was measured as described by *Geiger* et al. (13). Inhibition experiments in the presence of detergents were performed according to *Geiger & Fritz* (14).

Optimal reaction conditions (pH optimum, temperature stability, pH resistance) for porcine leukocyte elastase were determined as described by *Geiger & Fritz* (14).

Other methods, such as polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, isoelectric focusing, amino acid analysis, identification of the amino terminal amino acid residues and protein determination were performed as described in detail in the preceding paper (15).

Preparation of chelate Sepharose column

Chelating Sepharose 6 B (100 ml) was washed on a sintered glass filter (G 3) using 1.5 l dist. water. The Sepharose was than added to a glass column (70 × 2 cm) and loaded with copper ions at 4 °C by pumping through 190 ml of a 5 g/l copper sulphate solution. Thereafter the column was washed free of copper with dist. water and equilibrated with 0.04 mol/l Tris, 0.005 mol/l Na_2HPO_4, pH 8.2, containing 0.5 mol/l NaCl.

Isolation of porcine leukocyte elastase

Purification of leukocyte elastase was achieved by the following procedure (tab. 1).

1.Step: Leukocyte granule extracts were prepared by homogenization of leukocyte pellets (see starting material) in 30 ml of 0.2 mol/l sodium acetate buffer, pH 4.0 at 0 °C using a *Potter-Elvejhem* glass homogenizer. The extract was centrifuged at 30 000 *g* for 10 min at 4 °C and the pellet was rehomogenized and extracted with 3 separate quantities (30 to 50 ml) of buffer. The four extracts were combined (172 ml) and dialysed against 0.04 mol/l Tris, 0.005 mol/l Na_2HPO_4 containing 0.5 mol/l NaCl pH 8.2, overnight at 4 °C. The combined pellet extract was divided into four parts, and each part was subjected separately to chelating chromatography on copper chelate Sepharose, followed by ion exchange chromatography.

2. Step: The leukocyte granule extract (43 ml) was applied at 4 °C to a copper chelate Sepharose column (70 × 2 cm) equilibrated with 0.04 mol/l Tris, 0.005 mol/l Na_2HPO_4, pH 8.2 containing 0.5 mol/l NaCl at a flow rate of 36 ml/h. The column was developed immediately after application of the elastase solution with a pH gradient, formed by 650 ml 0.02 mol/l Na_2HPO_4 buffer, pH 7.7 containing 0.5 mol/l NaCl and 650 ml 0.1 mol/l sodium acetate buffer, pH 2.8 containing 0.5 mol/l NaCl at the same flow rate. Leukocyte elastase was eluted as shown in figure 1. Fractions of 10 ml were collected. The elastase containing fractions were combined and dialysed against 0.02 mol/l sodium acetate buffer, pH 7.0 containing 0.1 mol/l NaCl at 4 °C for 24 h.

3. Step: The elastase solution (60 ml) thus obtained was applied to a cooled (4 °C) CM-Sephadex C-50 column (35 × 2 cm) equilibrated with 0.02 mol/l sodium acetate buffer, pH 7.0, containing 0.1 mol/l NaCl (flow rate 23 ml/h; 8 ml fractions). The column was developed with a linear gradient from 0.1 mol/l NaCl in sodium acetate buffer at the same flow rate. The elastase-containing fractions (fig. 2) were combined, dialysed against 0.05 mol/l ammonium formate buffer, pH 7.0, lyophilized and stored at −30 °C. At this temperature the enzyme was stable for more than 2 years.

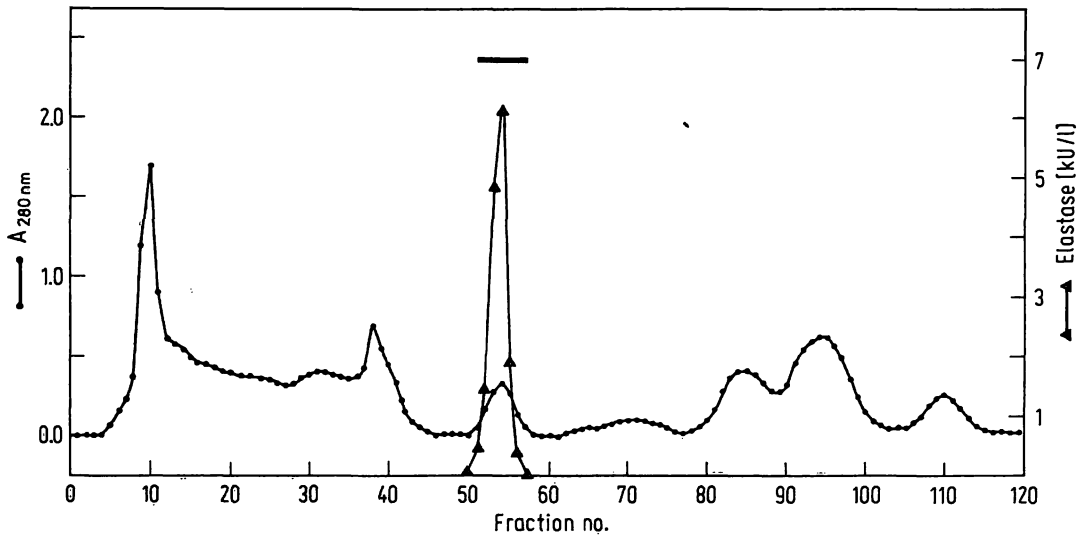

Fig. 1. Chromatography of porcine leukocyte elastase on copper chelating Sepharose.
Elastase activity was measured using MeOSuc(Ala)$_2$ProValNan as substrate (▲ — ▲). Protein concentration is given as absorption at 280 nm (●). For further details, see Methods.

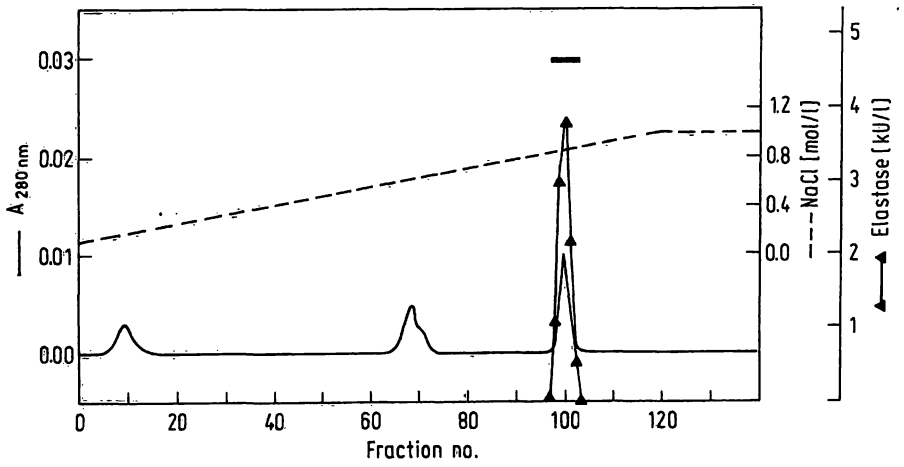

Fig. 2. Fractionation of the copper chelating Sepharose eluate on CM-Sephadex C-50.
Elastase activity was assayed by hydrolysis of MeOSuc(Ala)$_2$ProValNan (▲ — ▲). Protein concentration is given as absorption at 280 nm. For further details, see Methods.

Results

Isolation procedure

Porcine leukocyte elastase was purified using the procedure summarized in table 1.

Chemical and physical characteristics

Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate showed only one protein band corresponding to a molecular mass of 27 kDa (fig. 3).

On electrofocusing of a purified leukocyte elastase sample in an ampholyte gradient from 2 to 11, protein was detected only in the alkaline range of the gel.

N-terminal residues

Homogeneity of the leukocyte elastase preparation was checked further by analysis of the N-terminal amino acid residue. After one *Edman* degradation cycle, the phenylthiohydantoin derivatives of amino acids were identified by high performance liquid chromatography (16). The only residues found was isoleucine.

Amino acid composition

The amino acid composition from 3 runs on a Durrum analyser, based on a molecular mass of 27 kDa was determined. The data are listed in table 2.

Tab. 1. Purification of porcine leukocyte elastase. The data represent mean values of four preparations.

Step	Volume (ml)	Total protein (mg)[a]	Total activity (U)[b]	Specific activity (U/mg)	Purification factor
Leukocyte granule extract	43	180	202.1	1.12	1
Copper chelate chromatography	60	9.9	181.0	18.3	16
CM-Sephadex C-50 eluate	48	1.1	98.2	89.3	80

[a] based on the determination according to *Folin & Ciocalteau* (35).
[b] 1 unit = 10.2 ΔA_{405} per min.

Molecular mass determination

Molecular mass was determined by sodium dodecyl sulphate electrophoresis (see above) and was found to be close to 27 kDa.

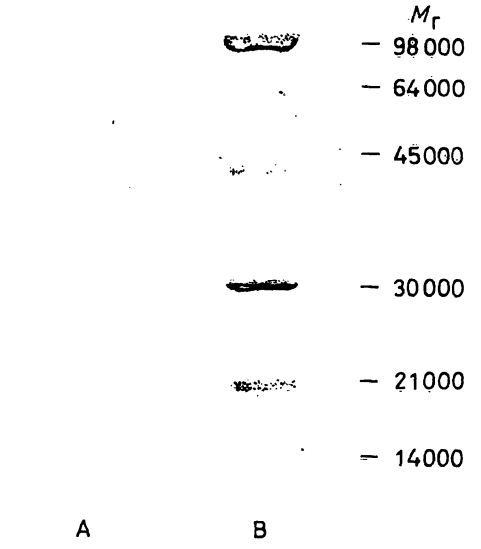

Fig. 3. Sodium dodecyl sulphate gel electrophoresis of porcine leukocyte elastase without reduction in gel A; molecular mass standards in gel B. For experimental details and M_r determination, see Methods. 10 µg of elastase were applied.

Tab. 2. Amino acid composition of human and porcine leukocyte elastase. The given values represent mol of residues per mol enzyme. The data represent mean values of three analyses.

Amino acid	Leukocyte elastase porcine[a]	human (18)
Asp	30	24
Thr	12	7
Ser	15	13
Glu	18	18
Pro	11	10
Gly	30	28
Ala	24	24
Cys	n. d.	6
Val	21	25
Met	2	2
Ile	5	11
Leu	16	20
Tyr	3	3
Phe	8	9
His	5	4
Lys	4	1
Arg	15	22
Trp	n. d.	2

[a] extrapolated to hydrolysis time zero
n. d.: not determined

Stability

The enzyme preparation obtained by the isolation procedure described above was stable at −20 °C for more than 2 years. At 4 °C no loss of activity was observed within two months if sodium azide was added at a concentration of 2 g/l.

The behaviour of leukocyte elastase at different temperatures and pH values is shown in figure 4 and figure 5, respectively.

pH optimum

For the hydrolysis of MeOSuc(Ala)$_2$ProValNan a pH optimum of 8.0 was found (fig. 6).

Kinetic constants

The K_m and V values obtained in the activity assays are given in table 3.

Inhibition studies

K_i values calculated from the inhibition curves obtained by titration of the leukocyte elastase with inhibitors are shown in table 4. Aprotinin, a potent

Tab. 3. Kinetic data for porcine leukocyte elastase and synthetic substrates. Kinetic constants were determined according to *Wilkinson* (10).

Substrate	K_m (mol/l)	V (μmol × min^{-1} × mg^{-1})*
MeOSuc(Ala)$_2$ProValNan	5 × 10^{-3}	89.3
Suc(Ala)$_2$ValNan	3 × 10^{-3}	29.8
Suc(Ala)$_3$Nan	4.5 × 10^{-3}	25.0

* Protein concentration was determined according to *Folin & Ciocalteau* (35). Human leukocyte elastase served as a standard.

Tab. 4. Dissociation constants of complexes between porcine leukocyte elastase and various trypsin inhibitors. For better comparison, K_i × 10^6 values are given in the table.

Inhibitor	Leukocyte elastase		
	Porcine K_i (μmol/l)	Human K_i (μmol/l)	
Soy bean trypsin inhibitor	0.4	0.2	
Turkey ovomucoid trypsin inhibitor	1.5	0.1	
Chicken ovomucoid trypsin inhibitor	2.5	1.2	
Lima bean trypsin inhibitor	5.4	2.1	
Eglin c (recombinant (27))	700	80	(29)
(isolated from leeches (29))	600	200	(28)

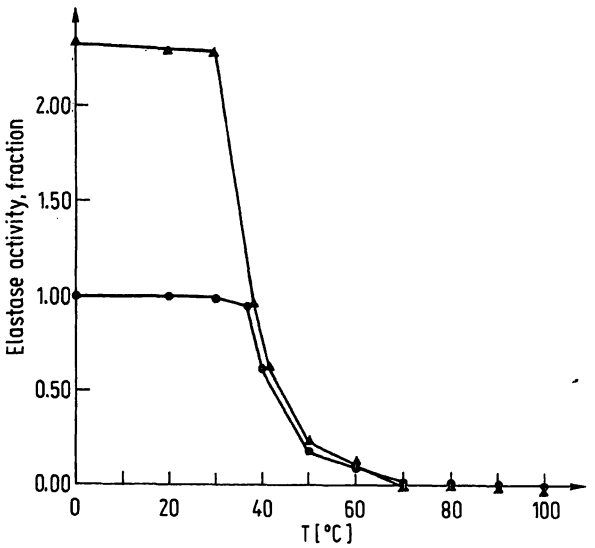

Fig. 4. Influence of temperature on the activity of porcine leukocyte elastase.
Elastase samples were incubated with (▲) and without (●) 1 g/l Triton X-100 at the given temperatures at pH 8.9; aliquot samples were removed after 15 min and assayed with MeOSuc(Ala)$_2$ProValNan as substrate.

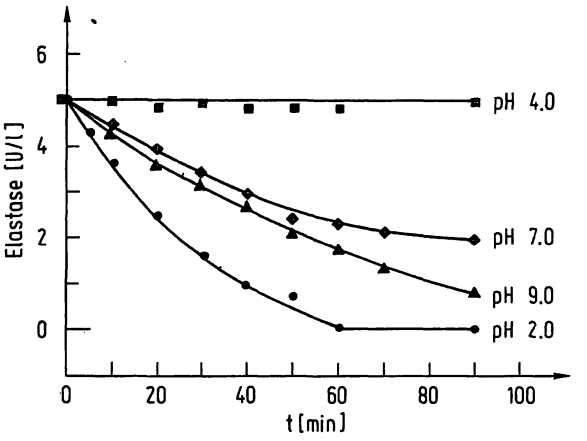

Fig. 5. Influence of pH on the activity of porcine leukocyte elastase. Elastase was incubated in solution up to 90 min at the given pH values; aliquot samples were removed at various time intervals and assayed with MeOSuc(Ala)$_2$ ProValNan.

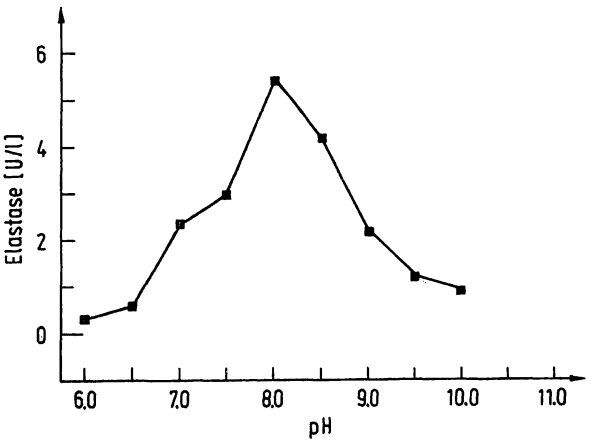

Fig. 6. pH optimum of porcine leukocyte elastase. Elastase activity was measured by hydrolysis of MeOSuc(Ala)$_2$-ProValNan.

inhibitor of trypsin, plasmin and kallikreins (17) caused no inhibition. For the inactivation of porcine leukocyte elastase with diisopropylfluorophosphate a bimolecular velocity constant of 2000 l × mol^{-1} × min^{-1} was determined at pH 7.2 and 25 °C. Results obtained by active site mapping experiments using active site labels are summarized in table 5.

Tab. 5. Kinetic constants for the inactivation of porcine leukocyte elastase by active site mapping reagents[a].

Affinity label	Concentration (μmol/l)	$t_{1/2}$[b] (min)	$10^{-4} \times k_{app}/l$[c] (l × mol^{-1} × min^{-1})
AcAlaAlaProAlaCH$_2$Cl	22	47	0.067
	44	27	0.058
	110	12	0.053
AcAlaAlaAlaAlaCH$_2$Cl	118	46	0.013
	177	29	0.014
ZPheCH$_2$Br	51	90	0.015
	126	25	0.022
Phenylmethylsulphonyl fluoride	115	88	0.007
	287	37	0.007

[a] inactivation experiments were conducted at 25 °C in 0.1 mol/l HEPES buffer, pH 7.5 which contained 0.5 mol/l NaCl and 100 ml/l in dimethyl sulphoxide.

[b] $t_{1/2}$ is the half time for pseudo-first-order inactivation at the indicated concentrations.

[c] k_{app}/l was calculated as described in l. c. (30/31).

Effects of detergents

The elastase-catalysed cleavage of MeOSuc(Ala)$_2$-ProValNan was significantly increased by addition of detergents to the test system: a 2 to 3-fold increase in enzymatic activity was observed in the presence of 1 g/l detergent (Triton X-100, Tween 20, Brij 35). Activation curves are shown in figure 7. A 2-fold increase in activity was also caused by porcine serum albumin.

Discussion

Leukocyte elastase has been isolated from granulocytes of several species, e. g. bovine, human, canine, horse (9, 18–24). For the purification of porcine leukocyte elastase we used a combination of isolation steps which were described formerly for the human enzyme: homogenization of the isolated granules and chromatography on copper chelate-Sepharose and CM-Sephadex C-50 under mild conditions (cf. table 1 and Results). In contrast to the human enzyme, porcine leukocyte elastase did not bind to aprotinin-Sepharose.

The purity of the enzyme preparation was proven by different criteria, such as quantitative end group analysis and sodium dodecylsulphate polyacrylamide gel electrophoresis.

The apparent molecular mass (27 kDa) of the porcine leukocyte elastase as estimated by dodecyl sulphate gel electrophoresis corresponds well with the molecular masses of carbohydrate-free serine proteinases of other species (18).

Electrofocusing experiments revealed that the porcine leukocyte elastase has a relatively alkaline isoelectric point, an observation which also holds true for the human granulocytic elastase (18).

An interesting feature of porcine leukocyte elastase is that the enzyme activity against synthetic substrates is significantly increased in the presence of detergents and serum albumin (see fig. 7), as also described for tissue kallikreins (14) and the sperm proteinases, acrosin (25).

The bimolecular velocity constant obtained (2000 l × mol^{-1} × min^{-1}) for the inhibition of leukocyte elastase by diisopropylfluorophosphate is in the range of data obtained for chymotrypsin (2700 l × mol^{-1}

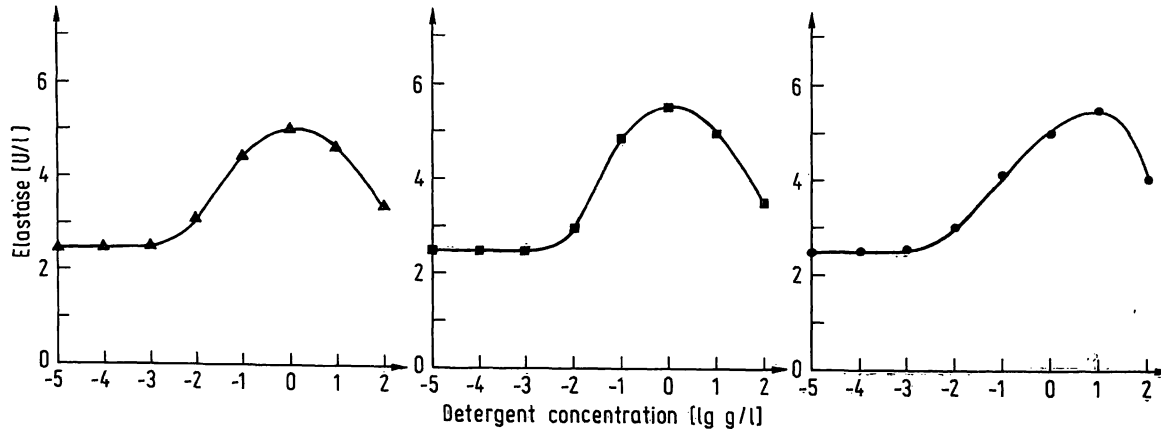

Fig. 7. Activation of leukocyte elastase by Triton X-100 (▲), Tween 20 (■) and Brij 35 (●). Enzyme activity was measured using MeOSuc(Ala)$_2$ProValNan as substrate.

$\times$ min^{-1} (26)), but differs from the values obtained for trypsin (300 l $\times$ mol^{-1} $\times$ min^{-1} (26)), or acetylcholinesterase (13 000 l $\times$ mol^{-1} $\times$ min^{-1} (26)).

The dissociation constants obtained for elastase and different *Kunitz* type inhibitors are in the range of 10^{-6} mol/l indicating that the enzyme is not so effectively inhibited by these inhibitors. In constrast, eglin, a very potent inhibitor of the human leukocyte enzyme, is also a potent inhibitor of the porcine enzyme. Aprotinin added to the test system up to 1 g/l showed no influence on elastase activity. Active site mapping reagents, such as chloromethyl ketones, ZPheCH$_2$Br and phenylmethylsulfonyl fluoride, showed medium inhibition of porcine leukocyte elastase.

The contribution of leukocyte elastase to pathophysiological situations is not finally understood. Based on experiments demonstrating a degradation of human lung elastin (32) an involvement of leukocyte elastase in the development of lung diseases, e. g. emphysema has been postulated (33). Furthermore it has been demonstrated that patients suffering from acute leukaemia and septicaemia have elevated leukocyte elastase-α_1-proteinase inhibitor levels in plasma (4). This last finding indicates a liberation of leukocyte elastase followed by an inactivation by endogenous α_1-proteinase inhibitor. But in the short time before elimination of leukocyte elastase via complex formation, the highly active enzyme may inactivate plasma proteins by nonspecific degradation as reported recently (3, 34). Existing correlations between elevated elastase-α_1-proteinase inhibitor levels and the depletion of clotting factors possibly support these postulates (4).

In order to investigate the possible contribution of leukocyte elastase in pathophysiological situations, the quantification of elastase is essential. Elastase is exclusively found in complexes with proteinase inhibitors (mainly α_1-proteinase inhibitor) in plasma, and this must be taken into consideration in any determination method for leukocyte elastase. Indeed a newly developed, sensitive enzyme immunoassay for human leukocyte elastase-α_1-proteinase inhibitor complexes became available recently (6). For studies in animals, e. g. pigs, a similar enzyme immunoassay using porcine leukocyte elastase has been developed[4]).

Acknowledgement

This work was supported by Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 207 grants LP-8 and LP-19. We wish to thank Prof. Dr. *H. Fritz* for many helpful discussion and comments and for the creative atmosphere he fosters in the department.

[4]) R. Geiger, S. Sokal and H. Fritz, manuscript in preparation.

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